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TITLE: TGFBI Regulation of Matrix Metalloproteinase-9 in Human  
Prostate Cancer Metastasis

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<b>13. ABSTRACT (Maximum 200 Words)</b> Matrix metalloproteinases (MMPs) facilitate invasion, growth factor activation, proliferation and angiogenesis during prostate cancer progression and are a target for anti-metastatic agents. Existing inhibitors of MMPs (MMPis) such as COL-3 and Prinomastat used in prostate cancer clinical trials are synthetic molecules and suffer from dose-limiting toxicities or lack of efficacy. This New Investigator research pursued an alternative course of MMP inhibition by studying growth factor-induced secretion of MMP-9 by TGFB1 in prostate cancers. Both MMP-9 and TGFB1 are clinically and experimentally associated with prostate cancer metastasis. TGFB1 upregulates cancer cell MMP-9 by mRNA stabilization. Since mRNA stability is usually regulated through binding proteins, we expressed fragments of the 3'UTR to determine if one or more fragment would be responsible for TGFB1 effects on mRNA half-life. We also began to use our MMP-9 3'UTR deletion constructs in vitro in cell lines and in vitro invasion assays to measure MMP-9 expression and inhibition of invasion as end points. Our attempts to test sequences for TGFB1-induced or repressed protein associations and identify such proteins are ongoing.				
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## Introduction:

The purpose of this project was to identify and test portions of the 3 prime untranslated region of the Matrix Metalloproteinase-9 (MMP-9) mRNA responsible for TGF $\beta$ -induced increases in mRNA stability which results in a growth factor-induced increase in MMP-9 protein expression. This was to be accomplished by generating constructs of the 3'UTR and comparing them with the entire MMP-9 coding sequence in transfection experiments to identify discrete regions of the MMP-9 3'UTR which mediate TGF $\beta$ 1 induction of increased MMP-9 mRNA half-life. We would analyze oligonucleotide sequences of the MMP-9 transcript for TGF $\beta$ 1-regulated protein-binding and ultimately characterize the binding proteins as regulators of mRNA stability. We will attempt to use portions of the 3'UTR to inhibit the increased expression of MMP-9 mRNA and protein and *in vitro* invasion.

## Body:

### Statement of Work:

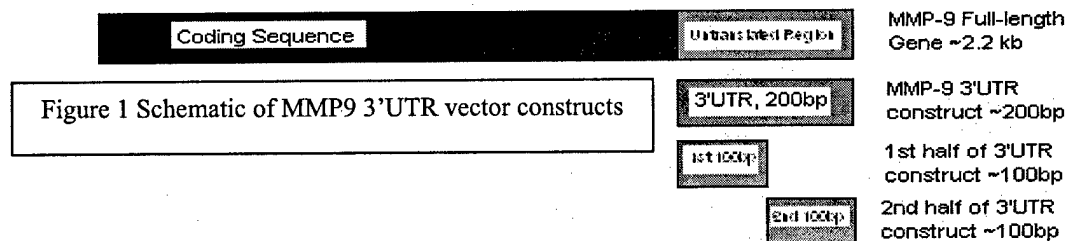
*Specific Aim 1. Map the 3'-UTR of the MMP-9 transcript for regions critical to maintenance of transcript stability*

*Specific Aim 2. Analyze sequences of the MMP-9 transcript identified in S.A. 1. for inhibition of *in vitro* invasion and for MMP-9 expression. Analyze sequences for TGF $\beta$ 1-regulated protein-binding.*

*Specific Aim 3. Characterize TGF- $\beta$ 1 regulated binding proteins and test these proteins as regulators of mRNA stability*

### Research Accomplishments:

Specific Aim 1: During the first two years, we designed and built constructs consisting of the complete MMP-9 coding sequence (CDS), the complete 3' untranslated region (3' UTR) of the MMP-9 gene, and the 3'UTR in one hundred base pair sections. Initially, attempts were made to clone PCR products of each of these constructs into both pBluescript and the pcDNA3.1 plasmid for anticipated future transfection assays in prostate cancer cells; however, we had difficulties with some of the fragments in one or the other plasmid, and thus we switched over completely to the pTargetT vector. This single vector allowed us to express mRNA *in vitro* and is has a CMV promoter for mammalian expression (Figure 1). We also switched the CDS+UTR fragment constructs made in year one to pTarget as well. Our pTargetT constructs have been sequenced to



confirm insert orientation and fidelity. We will use these constructs to test for binding of TGF $\beta$ 1-induced or repressed proteins from collected and frozen or fresh nuclear extracts. In addition, this vector is being used for tissue culture assays of invasion.

Specific Aim 2: To test our MMP-9 3'UTR deletion constructs for ability to reduce MMP-9 expression we transfected the Tsu-Pr1 cell line with pTarget plasmid and measured secreted MMP-9 by western blotting (Figure 2).

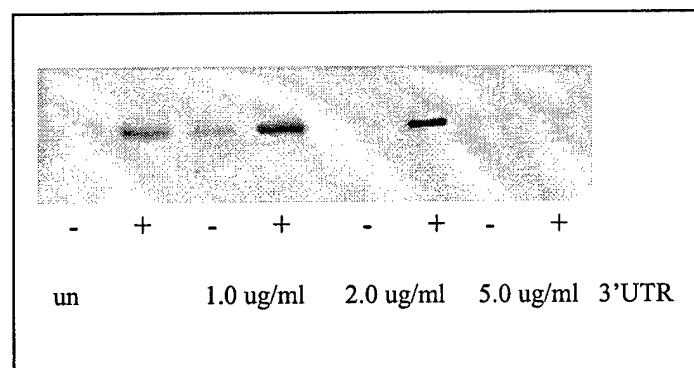


Figure 2. Shows a western blot depicting conditioned medium from transfected Tsu-Pr1 cells. The untreated lane (un) shows cells transfected with an empty plasmid, minus (-) or plus (+) TGF $\beta$ 1. There is a robust increase in MMP-9 expression. In the presence of 1.0 or 2.0 ug/ml 3'UTR-containing plasmid, TGF $\beta$ 1 still induces MMP-9; however in the presence of 5.0 ug/ml TGF $\beta$ 1, the response to TGF $\beta$ 1 is not apparent.

This Western blot suggests that transfection of the MMP-9 3' UTR produces some reduction in the ability of TGF- $\beta$ 1 to stimulate MMP-9 secreted protein levels in one cell line.

To test our MMP-9 3'UTR deletion constructs for ability to reduce MMP-9 secreted activity, we transfected the Tsu-Pr1 cell line with pTarget plasmid only of pTarget-3'UTR and cultured the cells in the presence or absence of TGF $\beta$ 1. Conditioned media was concentrated and analyzed by gelatin zymography (Figure3). This zymogram suggests that transfection of the MMP-9 3' UTR inhibits the ability of TGF- $\beta$ 1 to stimulate MM-9 secreted enzymatic activity.

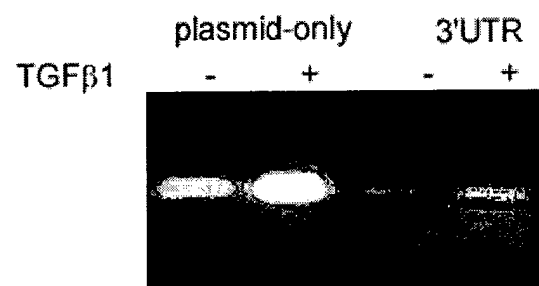
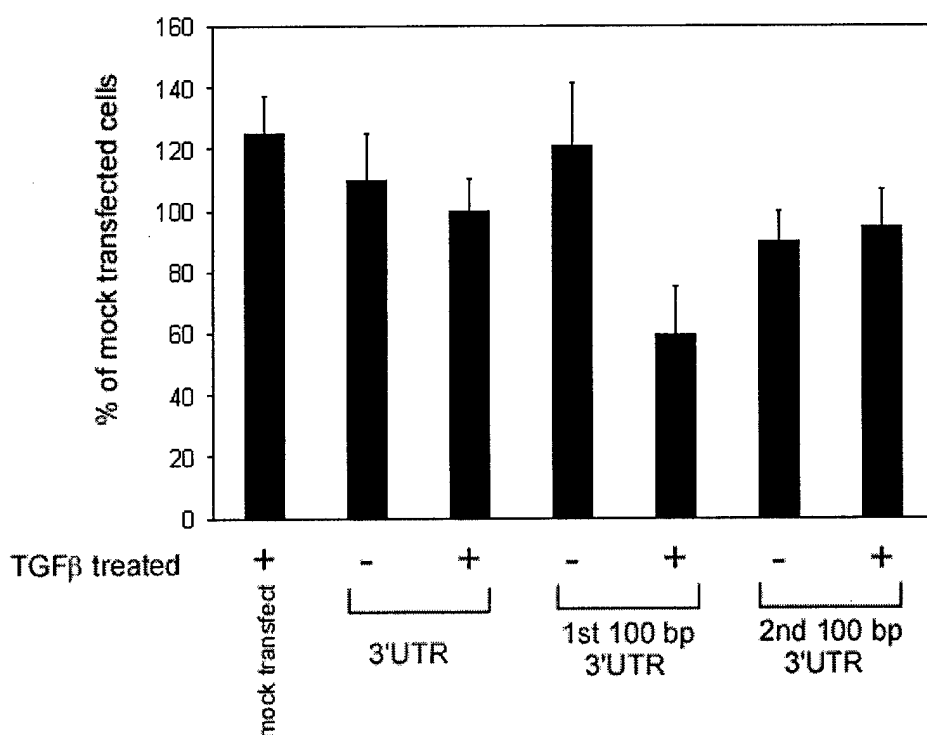


Figure 3. MMP-9 activity inhibited by over-expression of the MMP9 3'UTR.

Next, to test the MMP-9 3'UTR deletion constructs for ability to reduce in vitro invasion, we used the modified Boyden chamber assay transfecting with the 5 ug level of pTarget plasmid.

Figure 4. Tsu-Pr1 prostate cells were transfected over a 24 hour period in 35mm dishes using 5 ug/ml of plasmid DNA containing either pTarget with the whole MMP-9 3' UTR (3'UTR), pTarget with the first 100 base pairs of the 3'UTR (1<sup>st</sup> 100 bp 3'UTR) or pTarget with the 2<sup>nd</sup> 100 base pairs of the 3'UTR (2<sup>nd</sup> 100 bp 3'UTR). Twenty-thousand cells were then



applied to the top chamber of an *in vitro* invasion chamber containing growth factor reduced Matrigel basement membrane matrix. After a 48 period of invasion, cells which penetrated beneath the transwell membrane were stained with crystal violet and scored as cells per 20X field. Data is expressed relative to mock transfected cultures which were not treated with TGFβ (2ng/ml). The reduction in TGFβ-treated cells transfected with the first 100 bp UTR is significantly different than both the control and the untreated 100 bp cells ( $p < 0.05$ ).

This *in vitro* invasion assays indicates two main conclusions. First, TGFβ1 did not appreciably increase invasion in the mock transfected cells over untreated cells. This could indicate that the *in vitro* assay does not sensitively quantitate pro-invasive effects of TGFβ. Secondly, the whole 3'UTR, which reduced secreted levels of MMP-9 protein, did not significantly alter cellular invasion; however use of the first 100 base pairs of the 3'UTR region did. This indicates that in order to affect cellular invasion, a smaller segment of the UTR region is more effective.

An analysis of sequences for TGFβ1-regulated protein binding in this segment has not been completed.

Specific Aim 3. This aim has not been initiated. Unfortunately, beginning in 2001, laboratory progress was repeatedly slowed because of building construction/renovation.

This past year (5/03 to 5/04) was a no-cost extension of the original award. The PI, Inder Sehgal, relocated his research program to Louisiana State University from North Dakota State University. NDSU subcontracted remaining grant funds to LSU. Dr. Sehgal requested this DoD subcontract to begin July 1, 2003; which was approved by the DoD in December 2003 and therefore the subcontractee's efforts to rebuild all plasmid constructs, cell extracts and reagents pertinent to the Statement of Work was 5 months.

**Key research accomplishments:**

- Transfections of the MMP-9 3' UTR demonstrate some reduction in the ability of TGF- $\beta$ 1 to stimulate MMP-9 secreted protein and activity levels in one cell line.
- Transfections of 100 base pair sections of the MMP-9 3' UTR demonstrate differential ability to reduce in vitro invasion. The first 100 bp region shows in vitro efficacy while the second region and the entire 3'UTR do not.

**Reportable outcomes:**

A. Employment or research opportunities applied for and/or received based on experience/training supported by this award: Associate Professor faculty position, LSU Baton Rouge, College of Veterinary Medicine.

**B. Bibliography of Publications:**

*As suggested by the DoD reviewer, an agreement for submission of any future publications based upon use of supported constructs is made.*

Greiff, A., Fischer, W.M., and Sehgal, I. Paracrine communication between malignant and non-malignant prostate epithelial cells in culture alters growth rate, matrix protease secretion and in vitro invasion. *Clinical & Experimental Metastasis*, 19:727-733, 2002.

Sehgal, I., Forbes, K., and Webb, M.A. Reduced expression of MMPs, plasminogen activators and TIMPS in prostate cancer cells during repeated metastatic selection. *Anticancer Research*, 23: 39-42, 2003.

Forbes, K., Webb, M.A., and Sehgal, I. Growth factor regulation of secreted matrix metalloproteinase and plasminogen activators in prostate cancer cells, normal prostate fibroblasts and normal osteoblasts. *Prostate Cancer and Prostate Diseases*, 6:148-153, 2003.

Gillette, K.M., Forbes, K., and Sehgal, I. Detection of MMPs, TIMP-2, urokinase and PAI-1 within Matrigel and Growth Factor-Reduced Matrigel basement membrane. *Tumori*, 89: 421-425, 2003.

Personnel receiving pay from the research effort during the grant period (2000-2004):

Dr. Inder Sehgal (PI)

Ms. Andrea Grieff (graduate student, NDSU)

Ms. Karin Gillette (Research Technician, NDSU)

Dr. Xiaoyan Chen (Postdoctoral researcher, LSU)

Conclusions: TGF $\beta$ 1 may enhance MMP-9 post-transcriptionally by inducing protective elements that inhibit mRNA degradation. Competitive inhibition of these putative protective elements with overexpressed portions of the MMP9 UTR reduces some of the ability of TGF $\beta$ 1 to induce MMP-9.

“So what” section

The delineation of specific sections of the MMP-9 3'UTR which can decrease MMP-9 protein when overexpressed, could lead to the development of a specific method of targeting growth-factor induction of MMP-9 by prostate cancer cells. This could be a great improvement over the use of chemical MMP inhibitors, which are a class of compounds that have struggled to find success in human trials.

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